

Nasal vaccination using live bacterial vectors

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Abstract

Live recombinant bacteria represent an attractive means to induce both mucosal and systemic immune responses against heterologous antigens. Several models have now been developed and shown to be highly efficient following intranasal immunization. In this review, we describe the two main classes of live recombinant bacteria: generally recognized as safe bacteria and attenuated strains derived from pathogenic bacteria. Among the latter, we have differentiated the bacteria, which do not usually colonize the respiratory tract from those that are especially adapted to respiratory tissues. The strategies of expression of the heterologous antigens, the invasiveness and the immunogenicity of the recombinant bacteria are discussed. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Bacteria; Live vaccine; Intranasal; Heterologous antigens; Attenuation; Immunogenicity

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1. Introduction

Vaccination represents one of the most cost-effective public health tools to combat and sometimes

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eradicate infectious agents. Therapeutic treatment is usually far more expensive than prevention of disease. Although commercialized vaccines are generally effective, improvement in efficacy and safety, reduction in the numbers of administrations and the ease of administration are currently considered important challenges to be met for the next decades. Recent advances in biotechnology and in the understanding of the immune system have now made it possible to design new generation vaccines that may approach these goals. In particular, the use of recombinant micro-organisms to achieve optimal immune protection allows one to develop multivalent vaccines which require a reduced number of administrations compared to previous immunization schedules. In addition, and in contrast to most previous vaccines, live vaccines can induce mucosal as well as systemic immune responses when delivered by the oral or intranasal (i.n.) route. Among the various live vectors studied, bacteria have the additional advantage over viruses, that their genome is able to harbor many, in principle unlimited numbers of foreign genes, in contrast to viruses which encounter limits in the capacity to encapsulate foreign DNA. Recombinant bacteria have thus extensive possibilities to produce many different foreign antigens.

Most recombinant bacterial vaccine vectors have been designed to be administered by mucosal routes, such as the oral or i.n. route. Mucosal immunizations circumvent the need for specially trained personnel and instruments, avoid discomfort, and preclude the risk of disease transmission associated with parenteral administrations. Numerous studies using diverse antigens in various animal models have shown that particularly the i.n. route of administration can elicit a broad immune response, including serum, salivary, nasal, rectal and vaginal antibodies. These immune responses are often superior to those obtained after oral immunization [1]. Consequently, one of the goals of the World Health Organization is the development of new systems to deliver vaccine antigens to the respiratory tract. However, the use of engineered bacterial vaccine strains in humans requires a right balance between harmlessness, or the level of attenuation, and strong enough immunogenicity to stimulate protective immune responses. Two main approaches have been taken to meet these

requirements, the development of vaccine vehicles from generally recognized as safe (GRAS) bacteria and the development of vaccine strains through attenuation of bacterial pathogens.

2. GRAS bacteria

The GRAS status of a bacterial species represents an important advantage for its potential use as a live vehicle, since it implies the harmlessness of this particular strain as it has been extensively documented through its intensive use in humans. GRAS bacteria represent generally micro-organisms that are used in the food industry and/or that belong to the normal commensal microbial flora of healthy humans.

2.1. Food-grade bacteria

Most bacteria used in the food industry are poorly adapted for growth *in vivo* in a mammalian host, since they usually do not replicate within the host and have therefore a limited capacity to persist. This may have both advantages and disadvantages. It certainly contributes to the safety of such strains, but may have an impact on the immunogenicity of heterologous antigens produced by these strains. It is often considered that the production of heterologous antigens by such strains during *in vitro* growth is equivalent to a preloading of these bacteria prior to administration as a vaccine. The antigens are then presented to the immune system in a particulate form, which may increase their immunogenicity, and is thought less likely to induce oral tolerance than the same antigens presented in a soluble form [2].

However, although these bacteria generally do not colonize the host, little information is available about the fate of the recombinant bacteria within the host and about their interaction with the immune system and with the endogenous microflora. Recently, green fluorescent protein-producing lactic acid bacteria (LAB) have been developed [3–5] and used to trace the bacteria in the host. Fluorescent *Lactobacillus plantarum* were found to be efficiently phagocytosed by alveolar macrophages after i.n. inoculation in

mice [3], demonstrating that lactobacilli can be actively taken up by antigen-presenting cells and thus would be expected to properly present antigens for the induction of immune responses upon i.n. vaccination. Furthermore, fluorescent lactobacilli represent a powerful tool to evaluate the risk of DNA transfer among the bacteria of the commensal microflora.

2.1.1. *Lactococcus lactis*

Lactococcus lactis is one of the most advanced prototypes of non-invasive, non-colonizing bacterial vaccine vehicles. This LAB has been widely used in the food industry since immemorial times. Development of constitutive and inducible *L. lactis* gene expression systems for an efficient production of heterologous antigens made it possible to test this micro-organism as a live vector in animal models [6]. A high-level inducible expression system using the *Escherichia coli* T7 bacteriophage RNA polymerase has first been developed in *L. lactis* (pLET vectors). This permitted intracellular expression of various heterologous antigens at high levels (2–20% total soluble cell proteins), including tetanus toxin fragment C (TTFC), diphtheria toxin fragment B, and the 28 kDa glutathione S-transferase (Sm28GST) of *Schistosoma mansoni* [7–9]. pLET-derived vectors have also been designed to secrete heterologous antigens or to anchor them in the cell surface. Other expression vectors harbor constitutive low-strength promoters, which may be more suitable for antigens potentially toxic or insoluble when expressed at high levels. Such vectors have been used to express TTFC and Sm28GST at levels of 1–3% of total cell proteins [9,10].

Most immunological studies so far have been conducted with recombinant *L. lactis* producing TTFC [9–13]. Immune responses have been compared after i.n. or oral inoculation of recombinant strains which constitutively express cytoplasmic TTFC [7]. When C57BL/6 mice were i.n. immunized three times daily with 1×10^9 cfu at days 0, 14, and 28, elevated levels of TTFC-specific serum IgG1 and IgG2a were detected 7 days after the last administration. In contrast, oral immunization according to the same schedule failed to elicit a detectable anti-TTFC serum antibody response. To

reach antibody levels similar to those obtained after i.n. inoculation, two sets of three successive daily doses of 5×10^9 bacteria had to be given orally. In addition, mucosal anti-TTFC IgA responses were monitored in fresh fecal pellets. Fifteen days after immunization, the oral route led to a high but very transient response, whereas the nasal route provided a more sustained IgA level at least up to 41 days after immunization. Even though serum antibody titers obtained following oral immunization were lower than after i.n. immunization, the protective efficacy against a lethal toxin challenge ($20 \times \text{LD}_{50}$) given subcutaneously was similar. Colonization or invasion of the mucosa by the lactococcal vector appeared not to be necessary to elicit a strong immune response. Chemically inactivated recombinant bacteria given i.n. induced the same level of anti-TTFC serum antibodies as live bacteria [7]. Interestingly, however, a higher dose (5×10^9) of killed bacteria was required to achieve the same level of protection in mice against toxin challenge as the live bacteria (5×10^8) [14].

Upon immunization with recombinant *L. lactis*, only very low levels of antibody responses were mounted against the lactococcal proteins [14], rendering *L. lactis* immunologically as inert as synthetic microparticles. This finding suggests that *L. lactis* strains may be used repeatedly in the same host without inducing anti-vector immune responses. However, the particulate nature of the bacterial vector might affect the antigen presentation and uptake by the immune system. Intranasal co-administration of the recombinant *L. lactis* producing intracellular TTFC [8], together with the mucosal adjuvant cholera toxin failed to enhance anti-TTFC serum antibody responses, whereas it increased the response against lactococcal proteins [14]. In contrast, when cytokines, such as murine IL-2 or IL-6 were co-produced together with TTFC by the recombinant *L. lactis* strain, a 10- to 15-fold increase in anti-TTFC serum and enteric IgA titers was obtained upon i.n. inoculation in mice [15], whereas no increased immune response was observed against lactococcal proteins. In this case, the increase in TTFC-specific antibody titers was not observed when the recombinant lactococci were killed prior to i.n. administration, suggesting that the cytokines were secreted by live bacteria within the host.

2.1.2. *Lactobacillus*

Lactobacilli have also intensively been used in bioprocessing and for the preservation of food stuff. In contrast to *L. lactis* for which the studies conducted so far have used a single strain (MG1363), the *Lactobacillus* genus presents numerous vaccine candidate strains. Knowledge on the genetics of lactobacilli is more recent than that on lactococcal genetics. However, expression vectors, as well as chromosomal integration systems are now available [16–19]. Several heterologous proteins have been produced in the cytoplasm at up to a few percent of total cell proteins, secreted in the culture medium, or anchored in the cell surface of strains such as *Lactobacillus paracasei* LbTGS1.4, *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus zeae* ATCC393 and *Lactobacillus plantarum* 256 [16,17,20–22]. Inducible promoters are also available for lactobacilli. For instance, a nisin inducible expression system originally designed for *L. lactis* [23] was implemented in *L. plantarum* NCIMB8826 (Pavan et al., submitted for publication).

The particular attractiveness of some *Lactobacillus* strains resides in the natural adjuvanticity of their peptidoglycan layer [24]. In addition, certain lactobacilli can colonise the gut and exhibit probiotic health-promoting activities in humans and animals [25]. Although only few studies have been conducted with the nasal route, recent experiments [13] showed that similar to lactococcal strains, TTFC-producing lactobacilli induced antigen-specific serum IgG and local IgA responses after i.n. administration. Moreover, significant antigen-specific T cell responses were detected in cervical lymph nodes upon i.n. immunization with recombinant *L. plantarum* NCIMB8826 producing intracellular TTFC using either constitutive or inducible promoters (Grangette et al., manuscript in preparation).

2.1.3. *Staphylococci*

Staphylococcus carnosus and *Staphylococcus xylosus*, the two staphylococcal strains that have been tested for their potential as live vaccine vectors, are routinely used in starter cultures for meat and fish fermentation [26,27] and are commonly found in other food products such as in Mozzarella cheese [28]. These two species are non-pathogenic in mice upon oral or subcutaneous administration [29]. How-

ever, although *S. xylosus* is considered a commensal bacterium of the human skin [30], it has occasionally been involved in pyelonephritis [31] and endocarditis [32]. *S. xylosus* and *S. carnosus* present a low level of DNA sequence similarities to the pathogenic species *Staphylococcus aureus* and do not produce toxins, hemolysins, protein A, coagulase or clumping factor [33]. In addition, *S. carnosus* has very low extracellular proteolytic activity [33], which should permit stable surface display of heterologous proteins.

Powerful expression systems for surface display of recombinant proteins have been developed in both *S. carnosus* and *S. xylosus*. They are based on the use of the signal peptide and cell surface-binding regions of protein A from *S. aureus* (SPA), or the promoter, signal sequence and propeptide from a *Staphylococcus hyicus* lipase [34]. In addition, a gene fragment encoding the albumin-binding peptide BB derived from the streptococcal G protein, has been integrated in some constructs, so that the BB part can act as a reporter peptide of the recombinant proteins that can be detected by specific antibodies or fluorescence-labeled serum albumin. The BB part can also protect inserted peptides from C-terminal degradation by exopeptidases. This surface-display system has been used to display various epitopes, including *Plasmodium falciparum* peptides [35,36], a fragment of diphtheria toxin [37], epitopes from the human respiratory syncytial virus (RSV) [38,39], and the cholera toxin B subunit [40]. Antibody responses to the surface-displayed immunogens have been reported after i.n. administration of the recombinant bacteria. In an attempt to increase the immune response, vectors have been constructed that are based on the co-display of the immunogen with fibronectin-binding domains (FNBDs) from fibronectin-binding proteins of *Streptococcus dysgalactiae* and *S. aureus*. Since fibronectin is present in extracellular matrices and on epithelial cells, and is known to bind to various bacteria which carry fibronectin-binding proteins, the surface-display of FNBDs is expected to improve the capacity of the recombinant bacteria to adhere to respiratory epithelial cells. This may increase immune responses after i.n. administration. As expected, recombinant *S. carnosus* strains carrying surface-exposed FNBDs were able to bind to fibronectin and, after i.n.

administration, induced increased antibody responses to model immunogens co-displayed with FNBDs [41].

An alternative approach to improve immune responses upon i.n. administration of recombinant staphylococci is based on the co-exposure of a peptide (amino acids 50–75) of the cholera toxin B subunit with a model antigen [42]. The resulting recombinant strain elicited increased serum IgG and local IgA responses to the antigen upon i.n. immunization. This expression vector has recently been used for the surface-display of various peptides derived from the G protein of human respiratory syncytial virus subtype A [39]. When mice were immunized i.n. three times (days 0, 10, and 20) with 1×10^9 cfu, high levels of viral peptide-specific serum IgG1 and IgG2a were detected 20 days after the last administration, suggesting a balanced Th1/Th2 type response. When the mice were challenged with 1×10^5 tissue culture infectious doses 50 of the virus 10 days after the last immunization, lung protection was observed in approximately half of the mice.

In contrast to the lactococci, strong immune responses could only be induced by live bacteria. UV-irradiated or heat-killed staphylococci were poor inducers of immune responses, suggesting that some level of colonisation is required for the induction of immune responses [42].

2.2. *Streptococcus gordonii*

Among the commensal bacteria, streptococci may colonize the mucosal surfaces of humans and animals. Although mammals develop mucosal and serum antibodies after colonization by certain commensals [43,44], for yet unknown reasons, these antibodies do not appear to induce clearance. Therefore, it can be expected that mucosal colonization by recombinant strains derived from these micro-organisms results in the development of an immune response against the heterologous antigens, without necessarily ridding the host from the vector organism.

A variety of microbial antigens have been produced in commensal streptococci. The best studied species is *S. gordonii* Challis, formerly classified as *S. sanguis*. This strain was isolated from the human oral cavity and found to be naturally competent for

genetic transformation [45]. A stable expression system has been developed based on the chromosomal integration of foreign DNA encoding the heterologous antigen, and on the use of the M6 protein as a fusion partner for surface display [46–50]. M6 is a filamentous surface protein of *Streptococcus pyogenes* anchored in the cell wall by its C-terminal end. Recombinant *S. gordonii* have been constructed that surface-express heterologous proteins ranging in size from 15 to 441 amino acids. Several antigens of viral, bacterial, and eukaryotic origin, including the E7 protein of human papilloma virus type 16, the V3 domain of HIV-1 gp120, an allergen (Ag5.2) from hornet venom, ovalbumin, the surface F and H proteins of the measles virus, the B subunit of the *Escherichia coli* heat labile toxin and TTFC have been expressed using this system [10,19,46–49,51].

The recombinant vaccine strains were mainly tested in the mouse model, although recent experiments were also conducted in monkeys [19]. Recombinant *S. gordonii* is able to colonize various mucosal surfaces including the nasal cavity, as efficiently as the parental strain [46–48,19]. A single i.n. inoculation with 8×10^8 cfu of a recombinant *S. gordonii* strain producing on its surface the 204-amino acid allergen Ag5.2, resulted in colonization of the mouse pharyngeal mucosa for 10–11 weeks [48]. The production of the heterologous antigen Ag5.2 was stable during at least 11 weeks. Significant levels of Ag5.2-specific IgG and IgA were detected in serum and in the saliva and lung lavages, respectively. Systemic immune responses against a heterologous antigen displayed at the surface of *S. gordonii* could also be obtained after a single i.n. inoculation of 1×10^9 cfu of a recombinant strain producing the E7 protein of the human papillomavirus type 16 [46]. In all cases, i.n. inoculation with killed recombinant bacteria did not elicit a mucosal or systemic immune response, suggesting that colonisation by the recombinant *S. gordonii* strains is important for the induction of immune responses.

The colonization capacity of *S. gordonii* and its ability to induce local and systemic immune responses make this micro-organism an attractive bacterial vector. However, although classified as a commensal bacterium, *S. gordonii* has been associated with diseases, such as dental caries [52]

and endocarditis in humans [53]. Furthermore, the *S. pyogenes* M6 protein used for the surface display of heterologous antigens has long been considered a virulence determinant, even though the internal sequences are not included in the final constructs of recombinant *S. gordonii* strains. Therefore, there are still safety issues to be addressed before *S. gordonii* can be used as a vaccine vector in humans.

3. Attenuated bacterial strains

Microbial pathogens are particularly efficient to stimulate mucosal immune responses, since the majority of them colonize or enter through mucosal surfaces. Furthermore, natural infection with microbial pathogens usually induces strong mucosal and systemic immune responses, which may sometimes be protective against reinfection. As an alternative to GRAS bacteria, several attenuated pathogens have been developed and tested for their immunogenicity after mucosal administration, including i.n. administration. These can be divided into those micro-organisms that naturally do not colonize the respiratory tract, such as the enteric pathogens *Salmonella* and *Shigella*, and those that are particularly adapted to i.n. administration because they naturally do colonize the respiratory tract, including *Mycobacterium bovis* BCG and *Bordetella*.

3.1. Enteric pathogens

3.1.1. *Salmonella*

Attenuated *Salmonella* is the most intensively studied bacterial vector. More than 35 bacterial, 15 viral and 15 parasitic antigens have been expressed in this host [54]. Two *Salmonella* serovars have been most widely used as vectors, *Salmonella typhimurium* in mice and *Salmonella typhi* in humans. Attenuation of these strains by mutations of genes involved in metabolic pathways (*aro*, *pur*), in cAMP regulation (*cya*, *crp*) or in virulence (*phoP*–*phoQ*) has resulted in several avirulent strains that preserve various degrees of invasiveness and immunogenicity.

Initially designed to deliver antigens to the gut mucosa, attenuated *Salmonella* species have more

recently proven their ability to induce immune responses following i.n. administration as well, at doses generally 10-fold smaller than those used for oral administration [55]. *Salmonella* vaccine strains that have been i.n. administered at a dose of ca. 10^7 cfu can be recovered from the cervical lymph nodes, lungs, Peyer's patches and spleen for 40 days after administration [56]. *Salmonella* probably actively invades the M cells present in the nasal lymphoid tissue (NALT) in a way similar to the invasion of the Peyer's patches. The bacteria colonize the NALT within 1 day after inoculation and then disseminate via the draining lymph nodes within 5 days. Peak numbers in Peyer's patches and spleen are reached 10 days after i.n. administration [57].

Recombinant *Salmonella typhimurium* PhoP^c (constitutive activation of PhoP regulator) strains producing human papillomavirus type 16 virus-like particles or hepatitis B virus core antigen have been shown to induce antibodies specific of the heterologous antigen in i.n. administered mice [56,1]. In addition to systemic antibody responses, i.n. immunization also elicited IgA and IgG responses in respiratory and genital secretions. Interestingly, while i.n. immunization induced higher systemic and mucosal responses against the hepatitis virus B core antigen than oral, rectal or vaginal immunization, it induced lower responses against the *Salmonella* lipopolysaccharide (LPS), suggesting separate mechanisms underlying the immune responses against these two antigens [1]. *Salmonella typhimurium* was also tested as a mucosal vaccine vector for *Helicobacter pylori* antigens [58]. Mice i.n. immunized with recombinant bacteria producing the urease A and B subunits developed a mixed T-helper 1 (Th1) and T-helper 2 (Th2) immune response with induction of specific CD4⁺ T cells producing IFN- γ and IL-10, but not IL-4, in the spleen. Moreover, 60% of the immunized mice were protected against *H. pylori* infection after two i.n. administrations of 5×10^7 cfu of recombinant *Salmonella* at a 2-week interval.

Although these results are promising, an important drawback of the use of *Salmonella typhimurium* as an i.n. vaccine vector resides in the strong inflammatory reactions induced after i.n. application. An inoculum of 10^9 cfu of the *Salmonella typhimurium* PhoP^c strain was lethal when administered i.n., although it was well tolerated by the oral, rectal or

vaginal route [1]. This toxicity is probably related to *Salmonella* LPS signaling [54]. A *Salmonella typhimurium* strain producing detoxified LPS [59] may represent a potential solution to this problem. In addition, recombinant attenuated *Salmonella* PhoP^c strains, which have been extensively used and shown to be particularly immunogenic, contain a single point mutation which can revert at high frequency to the virulent phenotype [60]. New attenuated *Salmonella* strains harboring other mutations with a more stable phenotype are therefore needed and are now being evaluated [61–63].

In contrast to *Salmonella typhimurium*, which naturally infects rodents, cattle and primates including humans, *Salmonella typhi* is highly host-specific for humans. Although attenuated *Salmonella typhi* were in fact the first vaccine strains developed and tested in humans (as oral vaccines) [64,65], their strict host range has hampered the study of *Salmonella typhi* vaccine strains in mouse models. An important breakthrough has come recently through the work of Galen et al. [66] who showed that in contrast to oral inoculation, i.n. administration of attenuated *Salmonella typhi* producing TTFC alone or fused to the eukaryotic cell receptor binding domain of diphtheria toxin induced high titers of neutralizing anti-tetanus toxin serum antibodies.

Intranasal immunization with recombinant *Salmonella typhi* Ty21A displaying the hepatitis B surface antigen and the core protein of the hepatitis C virus on its surface has been shown to elicit high levels of serum antibodies against the two viral antigens, and the antibody titers were found comparable to those induced by intraperitoneal administration [67]. A recent study has suggested that the NALT of mice is a sufficiently inductive site to elicit immune responses against both the live vector and the heterologous antigen [68]. The relative contribution of NALT and lungs to the induction of serum antibody responses was determined by the use of an i.n. fractional-dose regimen (2.5 μ l administered i.n. every 15 min). Following this regimen, only a few bacteria were recovered from the lungs 1 h after inoculation of 1×10^9 cfu *Salmonella typhi*, whereas approximately 1 log of bacteria was still recovered from the NALT 18 h after inoculation. The persistence of the micro-organisms in the NALT was sufficient to elicit a serum IgG response.

3.1.2. *Shigella*

Attenuated strains of *Shigella* have also shown promise as live vaccine vectors carrying foreign antigens [69]. Attenuation was achieved by the deletion of genes involved in the biosynthesis of aromatic compounds (*aroA*), in intracellular and intercellular spread of the bacteria (*virG* also called *icsA*) or in the biosynthesis of guanine nucleotides (*guaBA*). In mice and guinea pigs, two i.n. administrations of 3×10^9 cfu of an attenuated $\Delta aroA \Delta virG$ *Shigella flexneri* strain producing two *E. coli* (ETEC) fimbrial antigens elicited specific secretory IgA in tears and IgA and IgG in the serum, although no serum anti-*Shigella* LPS IgG response was detected [70,71]. Barzu and co-workers [72,73] used the *S. flexneri* IpaC invasin as a carrier protein into which the C3 neutralizing epitope of the poliovirus VP1 protein was inserted. Recombinant *S. flexneri* SC602 was found to secrete the hybrid protein and to induce serum and local anti-C3 antibodies following a combination of systemic (1×10^8 cfu/mouse, three times at 15-day intervals) and i.n. administrations (5×10^6 cfu/mouse, three i.n. inoculations at 15-day intervals, beginning 15 days after the last intravenous immunization). However, in order to improve *S. flexneri* as a live mucosal vector, the amounts of hybrid proteins produced by the recombinant strain need probably to be increased. The C3 epitope represented only 3% of the hybrid protein, which itself was expressed at about 5 μ g per 1×10^8 bacteria. More recently, two i.n. administrations of 1×10^9 cfu of a $\Delta guaBA$ *S. flexneri* 2a strain producing TTFC was shown to efficiently protect guinea pigs against ocular challenge with wild-type *S. flexneri* 2a and to induce anti-tetanus toxin neutralising antibodies in serum [74]. These results show that the *S. flexneri* 2a vaccine candidate can deliver foreign antigens to the systemic immune system and serve as a mucosal *Shigella* vaccine.

3.2. Respiratory pathogens

Although attenuated enteric pathogens have certainly demonstrated their potential as live vectors for i.n. delivery of protective antigens, most of them show high reactogenicity/toxicity, and high doses have to be administered repeatedly to elicit optimal immune responses. Other bacteria, naturally adapted

to the respiratory tract have therefore also been explored as live attenuated vaccine vectors to be administered by the i.n. route.

3.2.1. BCG

Bacille Calmette–Guérin (BCG) was the first attenuated live bacterial vaccine developed and used in humans. It has been derived from a virulent *M. bovis* isolate by laboratory passages [75]. Originally administered orally, BCG is currently the most widely used human vaccine in the world and is the only available vaccine against tuberculosis. It can be given at birth, and its take is not affected by maternal antibodies. The low incidence of side-effects, its efficacy following a single-dose administration and its low cost have stimulated interest in its potential use as a delivery system for heterologous antigens [76].

Because of its persistence in vivo, recombinant BCG is thought to provide a continued and prolonged stimulation of the immune system by the foreign antigens. Furthermore, BCG possesses intrinsic adjuvant properties, especially for the development of cell-mediated immunity. Consequently, most of the heterologous antigens which have been produced in BCG, are intended to immunize against infectious agents whose protection is known to be mediated mainly by cellular immunity, such as against human papilloma virus [77], HIV [78], *Toxoplasma gondii* [79], *Leishmania chagasi* [80] or *Plasmodium yoelii* [81]. However, BCG is also able to elicit high antibody titers, and both cellular and humoral immune responses against a number of heterologous antigens have been observed in mice and sometimes in rhesus monkeys [78]. Some of these responses were shown to provide protection against the corresponding pathogen [79,81–86].

In most recombinant BCG strains, the heterologous antigens have been expressed from replicative plasmids, which have been shown to be stable in vivo. However, the presence on these plasmids of antibiotic-resistance genes as selectable markers could potentially contribute to the spreading of antibiotic resistance among other bacteria, including pathogenic mycobacteria. To overcome this problem, mycobacterial expression vectors are now available [87] that contain mercury resistance genes as the only selectable marker.

Various recombinant BCG strains have been tested in animals by administration via many different routes, including the i.n. route. Surprisingly, however, the i.n. route is less well documented than the others, although aerosol vaccination with BCG has long been known to be more efficient than vaccination by other routes in the protection of primates against tuberculosis [88]. Moreover, no adverse effects have been observed when BCG was given by aerosol to human volunteers, including young children, up to 10^5 organisms inhaled [89].

One of the earliest reports on the i.n. administration of recombinant BCG [82] describes the use of a BCG strain producing the outer surface protein A (OspA) from *Borrelia burgdorferi* as a membrane-associated lipoprotein using the *Mycobacterium tuberculosis* Mtb19 lipoprotein signal peptide [90]. A single i.n. dose of 1×10^6 cfu of this BCG strain induced a prolonged (more than 1 year) protective systemic IgG response and a highly sustained, low-titer serum IgA response against OspA. Furthermore, the i.n. administration induced high and persistent (until 22 weeks after immunization) levels of OspA- and BCG-specific IgA spot-forming cells in the lungs, indicative of a strong secretory IgA response. Intraperitoneal immunization with the same amount of bacteria did not result in a measurable anti-OspA serum IgA titer or in OspA- and BCG-specific IgA spot-forming cells. Oral delivery of 1×10^7 recombinant BCG elicited low levels of serum and gastrointestinal OspA-specific IgG and IgA, respectively. Finally, OspA-specific IgA were also found in vaginal washes upon i.n. but not upon intraperitoneal administration of the recombinant BCG, and were still detectable after 19 weeks.

The production of OspA as a membrane-associated lipoprotein was 100- to 1000-fold more immunogenic than the non-lipidated form produced either in the cytoplasm or as a secreted protein [90]. However, i.n. immunization with recombinant BCG can induce high levels of antigen-specific antibodies even in the absence of secretion or of a lipid moiety attached to the foreign antigen. This was demonstrated by the use of a BCG strain producing the glutathione S-transferase (Sh28GST) from *Schistosoma haematobium*. After a single i.n. administration of 10^6 recombinant bacteria to mice, strong and sustained systemic and mucosal immune responses

against Sh28GST were obtained [91]. The serum IgG response was at the same level as that obtained after systemic immunization. However, in contrast to the systemic immunization, i.n. delivery induced a mucosal IgA response against Sh28GST as well. Interestingly, the serum IgG response against Sh28GST, obtained after i.n. administration of the recombinant BCG strain was substantially higher than that obtained against Sm28GST after i.n. administration of a recombinant BCG strain producing Sm28GST, although both antigens share approximately 90% identical amino acids and were produced at the same levels by their respective recombinant BCG strains [91,92]. In addition, both BCG strains induced the same levels of their respective anti-GST antibodies when administered intraperitoneally, suggesting that even slight variations in the primary sequences of the antigens may have profound effects on the levels of immune responses specifically elicited by the i.n. route.

Since BCG can be given at birth, the use of this micro-organism for vaccination of infants against serious infectious childhood diseases such as measles virus pneumonia could be very effective. Intranasal administration of a recombinant BCG strain producing the measles virus N nucleoprotein in the cytoplasm was found to provide protection against virus challenge in infant rhesus macaques [93]. No clinical signs or lesions were observed following i.n. immunization of the monkeys, whereas local suppurative skin abscesses and regional lymphadenitis were observed in 50% of the intradermally-inoculated animals. Both routes gave rise to a weak proliferative and cytotoxic T cell response to the measles virus, but no protein N-specific serum IgG or IgM response was detected. Nevertheless, the vaccinated monkeys showed a significant reduction of lung inflammation after challenge with the virus. Moreover, virus titers in lymph nodes were lower, and the duration of the nasopharyngeal viral shedding was shorter, suggesting that specific T cells were primed by the i.n. vaccination, thus preventing virus-induced lung pathology.

Attempts to protect against human papilloma virus-induced tumors by i.n. vaccination with recombinant BCG have also recently been undertaken. The L1 late protein and the E7 early protein of the virus have been produced in BCG [77], and the immune

responses were tested after administration of 2×10^6 cfu by the intravenous, subcutaneous and i.n. routes. Intranasal immunization gave lower antibody titers but higher T cell proliferative responses than the other routes. However, regardless of the route, the magnitude of the observed responses was less than that elicited by a protein/adjuvant vaccine, and was not protective in a tumor challenge model. Nevertheless, upon i.n. immunization with the recombinant BCG strain, mice were primed effectively for subsequent recall of immunity. Since protective immune responses against virus-like particles may be predominantly directed to conformational epitopes, it is possible that the viral proteins presented by the recombinant BCG did not adopt a native configuration, which thus may be responsible for the low responsiveness.

3.2.2. *Bordetella pertussis*

Bordetella pertussis, the etiologic agent of whooping cough and a strictly respiratory pathogen, has recently been developed into a bacterial vector for i.n. vaccination [94,95]. Virulent *B. pertussis* colonizes the human respiratory tract very efficiently and induces a strong and protective immune response after natural infection in humans. Although it colonizes the mouse respiratory tract much less efficiently than the human respiratory tract, mice can nevertheless be used as a model system to study the mechanisms of immunity to *B. pertussis*, since protection against aerosol challenge of adult mice correlates well with vaccine efficacy in children [96]. Efficient colonization by *B. pertussis* depends on the production of numerous virulence-associated adhesins and toxins [97]. Among the adhesins, filamentous hemagglutinin (FHA) is the most important one [98]. It is the major surface-associated and secreted protein of *B. pertussis*. Consequently, FHA has been used to carry heterologous antigens to the bacterial surface [94] or/and to secrete them into the extracellular milieu (Coppens et al., submitted for publication). To genetically stabilize the recombinant *B. pertussis* strains even in the absence of selective pressure, the foreign genes have been integrated into the bacterial chromosome at the FHA locus.

FHA has been shown to express immunostimulatory properties. Serum antibody titers raised against protein antigens incorporated into liposomes

and delivered by the i.n. route were greatly increased when the liposomes also contained small amounts of FHA [99]. These immunostimulatory properties together with the high levels of FHA production and secretion by *B. pertussis* make this molecule an attractive carrier for heterologous antigen presentation at the bacterial surface. Bacterial (Coppens et al., submitted for publication), viral (S.A., unpublished observations) and parasite [94] antigens have been fused to FHA and presented via recombinant *B. pertussis* to the respiratory mucosa.

A single i.n. administration with 5×10^6 cfu of a recombinant *B. pertussis* strain producing FHA fused to the *S. mansoni* antigen Sm28GST resulted in local FHA- and Sm28GST-specific IgA and IgG responses in the bronchoalveolar lavage fluids of mice, 4 weeks after immunization [94]. The elevated levels remained constant at least for 8 weeks. However, the i.n. administration did not result in detectable serum anti-Sm28GST antibodies, strongly suggesting that those detected in the bronchoalveolar lavage fluids were produced locally. Despite the absence of detectable Sm28GST-specific serum antibodies, a single i.n. administration of the recombinant *B. pertussis* strain elicited immunological memory, since high levels of anti-Sm28GST serum antibodies were found when the mice were subsequently boosted with the purified antigen or by parasite challenge [100]. These antibodies were of the IgG1, IgG2a, and IgG2b isotypes, suggesting a mixed immune response. Interestingly, a single i.n. administration of this recombinant *B. pertussis* strain followed by a booster dose with the purified Sm28GST antigen resulted in low (40%) but significant protection against *S. mansoni* infection.

Early local production of proinflammatory cytokines, such as tumor necrosis factor α , interleukine 6 and transforming growth factor β was associated with i.n. administration of the recombinant *B. pertussis* [101]. However, this proinflammatory cytokine production was transient and lasted for less than 1 week following infection.

Since *B. pertussis* is a human pathogen, attenuation is required before it can be considered as a live vector for vaccination. Initial attempts to genetically attenuate *B. pertussis* consisted in the inactivation of the *aroA* gene. Deletion of *aroA* resulted in strong attenuation, and the mutant strain failed to colonize

the lungs of mice even if the bacteria were still able to produce important virulence factors such as pertussis toxin and FHA [102]. However, the mutant *B. pertussis* strain was poorly immunogenic, although it was able to prime mice, since immunized animals showed a rapid anti-*B. pertussis* humoral antibody response after exposure to wild-type challenge.

A second strategy of attenuation has been based on the increasing knowledge on molecular aspects of *B. pertussis* pathogenesis [97]. In this approach specific virulence factors were targeted in order to diminish the virulence but to maintain the colonization potential of *B. pertussis* and therefore its immunogenicity. The deletion of the genes encoding pertussis toxin, the major virulence factor of *B. pertussis* [103], has led to a highly attenuated strain, as evidenced by a strong reduction in lung inflammation [104] and in lymphocytosis. Moreover, using the coughing rat model of pertussis, Parton et al. [105] have shown that a mutant *B. pertussis* strain deficient in pertussis toxin production was inactive in cough production.

Despite its strong attenuation, the toxin-deficient strain was still able to colonize the respiratory tract of mice almost as efficiently as the parent strain. In addition, a single i.n. administration of the mutant strain provided protection against a challenge infection with wild-type *B. pertussis* [95]. This protection was equivalent to that provided by vaccination with commercially available pertussis vaccine or infection with the wild-type strain. Surprisingly, the deletion of the pertussis toxin genes resulted in increased immunogenicity against FHA with an approximately fivefold increase in serum anti-FHA IgG. Interestingly, deletion of the pertussis toxin genes from the chromosome of a recombinant *B. pertussis* strain caused also an increase in immunogenicity against the heterologous antigen fused to FHA. A single i.n. administration with 5×10^6 cfu of a recombinant attenuated *B. pertussis* strain producing Sm28GST resulted in the production of anti-Sm28GST serum antibodies which was not detectable after administration of the recombinant non-attenuated strain [100]. Moreover, significant protection against *S. mansoni* challenge with a reduction in worm burden of approximately 60% was observed [95], indicating that a single i.n. vaccination with a

recombinant attenuated *B. pertussis* strain can efficiently protect against homologous and heterologous diseases.

Intranasal administration of live attenuated *B. pertussis* can also elicit long-lasting specific antibody responses against FHA in the genital tract of female mice [106]. Anti-FHA IgA and IgG could be measured in genital tissues, both in the vagina and in the uterus, 28 days after a single i.n. administration with 5×10^6 cfu of attenuated *B. pertussis* and lasted for at least 14 weeks. This observation suggests that attenuated *B. pertussis* may also be a promising vector to induce antibody responses against antigens from sexually transmitted pathogens fused to FHA.

4. Conclusion

Live recombinant bacteria appear to be attractive and promising vehicles of vaccine delivery able to induce both humoral and cellular immune responses. Colonizing bacteria, such as attenuated pathogens, are generally more potent vectors than non-colonizing micro-organisms, such as certain lactic acid bacteria. Among the former ones, attenuated vectors derived from respiratory pathogens are particularly well adapted, since they present the advantage of colonizing the respiratory tract and thereby eliciting long-lasting immunity. Compared to other routes of mucosal immunization and to other vaccine vehicles, reduced numbers of inoculations and of vaccine doses are usually sufficient to elicit strong immune response due to the prolonged persistence of the bacterial vector. Moreover, in contrast to systemically administered vaccines, i.n. administered recombinant live bacteria induce generally strong mucosal immune responses in the respiratory tract and also at distant mucosal sites. Since recombinant bacteria may contain many different foreign genes, they are particularly useful for the design of multivalent vaccines able to protect against several pathogens simultaneously. Immune responses induced in the respiratory tract may disseminate throughout other mucosal surfaces. Therefore, this vaccine strategy may protect against pathogens, which naturally infect their hosts via different sites such as the genital tract. In addition, the strong systemic immune responses

induced by i.n. vaccination may help to control the dissemination of infection.

Although live bacteria offer many advantages to present heterologous antigens to the immune system, application of such vaccines in humans requires important safety issues to be addressed. In particular, the harmlessness of live recombinant bacteria in young children, in the elderly, and in the immunocompromised population has to be evaluated. Vaccine strain stability and potential genetic recombination with commensal or pathogenic micro-organisms need further investigations.

Increased knowledge in pathogenesis of the virulent parent strains of attenuated bacteria will bring further information to help optimizing attenuation of recombinant bacteria without reducing their immunogenicity. A better understanding of the involvement of bacterial virulence factors in the induction of immune responses is also of utmost importance. The toxicity of some virulence factors can be reduced by genetic alterations, and the immunomodulatory properties of some bacterial components can be used to elicit the desired type of immune response required to protect against the heterologous pathogens. Although attenuated bacteria have not yet been used as nasal vaccines in humans, they have been extensively studied for use in veterinary medicine (for a recent review, see Ref. [107]). The information gathered by the veterinary use of attenuated bacterial vaccines administered i.n. will undoubtedly be very useful for the application of this concept in humans.

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